EARTHWORM AS A MODEL ORGANISM TO IDENTIFY MITOGENIC AND ANTIMITOTIC COMPOUNDS FROM PLANTS

A SYNOPSIS

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1. INTRODUCTION

Earthworm species, *Eudriluseugeniae*, has been developed here as a simple *in vivo* model organism to evaluate the potential of unknown mitogenic and antimitotic compounds from plants. The regeneration potential of amputated earthworm has been used in our studies. Moreover, the usage of coelomic fluid of earthworm as nutrients in plant tissue culture medium was studied.

1.1.Mitogenic compounds

Mitogenic compounds make cells divide, which is called mitogenesis. They're very important in the field of health sciences and medicine. These compounds help cells grow and fix themselves. They make cells go through the cell cycle, which includes copying DNA and dividing. These signals are important for making new cells and tissues. They also help cells become different types. During the wound healing, tissue repair or regeneration process mitogenic compounds help create new cells. Scientists are studying these compounds to find new drugs. These compounds also help stem cells divide and repair tissues. In neuroscience, they study how mitogenic compounds can treat brain diseases. In the immune system, these compounds help immune cells grow and fight germs. Knowing about mitogenic compounds is important for healing wounds and treating diseases with abnormal cell growth. These compounds play major role in understanding cell division and in medical sciences**.**

1.2. Antimitotic compounds

Antimitotic compounds stop or disturb cell cycle’s essential step called mitosis. In all eukaryotic organisms, mitosis is crucial for tissue growth, development, and upkeep. Studying antimitotic compounds is very important in medicine because they're crucial for research related to health sciences. Compounds like vinca alkaloids and taxane are often used in cancer treatment to control cell division by affecting microtubules. These compounds help us understand how cells divide and contribute to fighting diseases. Scientists are always looking for new compounds that work better and have fewer side effects. Colchicine, another known, well documented antimitotic compound, arrests cell division by interacting with tubulin proteins and disruptsthe development of microtubules. Colchicine-treated cells are normally arrested at metaphase of mitosis. Knowing how antimitotic compounds work helps us to understand more about the cell division. Antimitotic drugs also help by slowing down immune cell growth and reducing swelling, which is helpful in treating autoimmune diseases where the immune system is too active. These compounds are super important in health research, especially for treating cancer and birth control. Researchon antimitotic compounds helps to find new compounds and how they can help us stay healthy.

1.3.Earthworm

Earthworms are frequently used as model organisms in scientific research due to their unique biological characteristics such as ease of maintenance, and applicability to a wide range of scientific disciplines. Earthworms, which belong to the Annelida phylum and many difference species, are available. We have used the species namely *Eudrilus eugeniae*.

Earthworms are known for their impressive ability to regenerate lost body parts. This makes them valuable for studying tissue repair and regeneration. The clitellum region, a special part of earthworms, is present in the anterior part of the worm, which is essential for regeneration in *E. eugeniae* species. The cells in the clitellum region act like stem cells and support the growth of new cells, tissues and organs. Earthworms, being simple invertebrates with a basic nervous system and limited senses, do not require the same ethical clearance as more complex animals like mammals. These worms are easy to handle and care for in laboratory settings, making them a convenient choice for research. Amputated earthworm species of *E. eugeniae*, the anterior part which retains clitellum, will survive and regenerate into complete worm. However, the posterior part, without clitellum, will not survive or regenerate. This regeneration ability of earthworm is used in our experiments to identify mitogenic and antimitotic compounds from plant samples.

The clitellum region is considered to have stem cells, which is secreting a protective layer and providing support and nutrients to the wounded cells or amputated region. Amputated earthworms, that retain clitellum region, regenerate lost segments more effectively at the wounded region by development of blastema. These cells in blastema can divide and become specialized to replace the lost parts. Earthworms, being simple invertebrates with a basic nervous system and limited senses, do not require the same ethical clearance as more complex animals like mammals and birds. Earthworms are relatively easy to handle and care for in laboratory settings, making them a convenient choice for research. It takes approximately 7 days for an earthworm to regenerate its functional anus.

It is a tremendous challenge for basic preclinical and clinical research to evaluate anticancer or antimitotic drugs. Over decades, researchers have developed well-proven, reliable *in vitro* and *in vivo* methods to evaluate drugs. For most anticancer agents, the initial step of evaluation is using the cell culture technique. The use of these standardized experimental methods is time-consuming and costly. Given that it is practically impossible to test large quantities of new anticancer agents in vivo.The most important function of in vitro experiments is to select promising candidates for further testing and to gain insights of their mode of action.

1.4.Coelomic fluid

Earthworms, which are a type of segmented worms, have a fluid called coelomic fluid in their body cavity.The coelomic fluid of earthworms is crucial for their physiological processes. It is located in their coelomic cavity, which is a large area filled with fluid surrounding their internal organs. This fluid is very important for maintaining their overall balance and responding to changes in the environment. It keeps their body tissues moist, helps with movement inside their burrow and through the soil, and acts as a lubricant. Additionally, it aids in the transportation of nutrients, gases, and waste products within their body cavity, which helps circulate nutrients between different segments. The coelomic fluid also contains coelomocytes cells and other molecules that are part of the earthworm's immune system. These components help the earthworm recognize and neutralize harmful substances or foreign particles. Changes in the composition or characteristics of the coelomic fluid can affect how earthworms behave. For example, they may release it as a defense mechanism when they're stressed or disturbed. The specific composition and functions of the coelomic fluid can vary among different earthworm species and can be influenced by environmental factors.

We have used earthworm, *E. eugeniae*, as a model organism to identify mitogenic and antimitotic compounds from plant extracts. The regeneration ability of amputated earthworms was exploited in our experiments. Mitogenic compounds promote regeneration whereas antimitotic compounds inhibit regeneration from the amputated region. Besides, we have studied the benefits of coelomic fluid of earthworm in plant tissue culture medium by using the model plant *Nicotiana tabacum*.

2. LITERATURE REVIEW

2.1. Inexpensive simple *in vivo* model system:

Invertebrate organisms are used in laboratory studies for diseases like Parkinson's, endocrine dysfunction, and muscle dystrophy, wound healing, and more. Despite their undeveloped organ system and immune system, they offer benefits like a brief life cycle, small size, and simple anatomy, allowing for a large number of invertebrates to be studied with less ethical issues [1] (Doke&Dhawale 2015).

Earthworms are important model organisms across various scientific disciplines, such as ecology, environmental science, neuroscience, cell biology and genetics. [2] Rajamanikkam et al., (2019 a) reported that earthworm *E. eugeniae* can be used as simple inexpensive *in vivo* system to evaluate unknown antimitotic compounds. They showed that the medicinal plant, *A. calamus* contains a compound that inhibits cell division. Hence the earthworm regeneration assay could be a cost-effective alternative system for screening such compounds. This system is easily adaptable by researchers worldwide, as it does not require expensive infrastructure or ethical approvals in many countries.

Previous research conducted in our laboratory involved extraction of*Acorus calamus* rhizomes with sterile water to identify water-soluble antimitotic compounds. Aqueous-extract of *A. calamus* rhizomes inhibited the cell division efficiently in onion root tips and in cell culture assys (MTT assay with MCF-7 breast cancer cell line). Interestingly this aqueous extract inhibited the regeneration of amputated earthworms. These results clearly suggested that earthworm could be used as simple *in vivo* system to screen compounds for their antimitotic potential [2] (Rajamanikkam et al., 2019 a).

2.2. Pharmacological importance of *Acorus calamus*

A. calamus rhizome had been shown to be beneficial in preventing renal stone formation[3] (Ghelani, et al., 2016). Previous study on the ethanol extract (80%) of *Acorus calamus* leaves showed an anti-inflammatory effect on albino rats. Ethanolic extract of *A. calamus* leaves accelerated wound healing activity, supporting its use in topical wound treatment and management [4] (Jain et al., 2010 b).Ethanolic extract of *Acorus calamus* does not pose any potential toxicity issues in both acute and chronic toxicity studies[5] (Shah, et al., 2012).In our laboratory, [6] Rajamanikkam et al., (2019 b) fractionated the aqueous extract of *A. calamus* with different organic solvents and found out that ethanolic fraction induced blastema development in amputated earthworms. Hence, earthworm species *E. eugeniae* could also be used to screen mitogenic compounds from unknown sources.

2.3. Bioactive compounds from *Musa paradisiaca* pseudostem

Musa paradisiaca, a type of banana tree, generates a considerable amount of waste biomass after the harvest period. The pseudo-stem, responsible for providing nutrients to the fruit, becomes waste after the harvest. Approximately 220 tons of this biomass waste is produced per hectare of banana plantation. Currently, this waste is disposed of in rivers, lakes, or landfills, which poses a severe risk to the environment [7] (Shah et al., 2005).

The pseudostem exudate of *M. paradisiaca* L. demonstrated the highest cytotoxicity against HCT-116 and HepG-2 tumor cell lines, with an IC50 of 29.4 µl for both. This indicates that the exudates could potentially be used for medicinal purposes because of its biologically active compounds, including phenols, alkaloids, saponins, flavonoids, and glycosides. Additionally, the crude exudate of *M. paradisiaca* L. exhibited antioxidant, antitumor, and antibacterial properties, which further supports its traditional use in treating various diseases[8] (Abdel Ghany, et al., 2019).

2.4. Earthworms' Coelomic fluid on plant growth

The cold stress method is recommended for collecting CF from *E. eugeniae* as it doesn't alter the optimal activity of proteins, enzymes, nutrients, and small molecules in the liquid. The concentration of CF is beneficial for seedling growth. The growth enhancement of *V. radiata* is believed to be due to the synergistic act of volatile compounds, nutrients, hormone-like compounds, enzymes, and proteins in the CF of earthworm *E. eugeniae*[9] (Nadana et al., 2019).CF extruded by cold method was found to enhance seed germination and growth compared to CF prepared by other methods. This is possibly due to the optimal pH of CF (extruded by cold method) and its enzymatic activities during the seed germination. The presence of heneicosane and squalenein cold CF might have promoted the seed germination and plant growth. Cold CF triggered seed germination ahead of time and induced more phenolic and antioxidant compounds during the germination of green gram seeds [10] (Nadana, et al., 2020 a).

In our laboratory we discovered that CF of earthworm *E. eugeniae* can boost the growth of rice seedlings and make them more resistant to a fungal pathogen known as *R. solani*[11] (Nadana et al., 2020 b). Transgenic rice plants expressing PR proteins found to be resistant towards this fungal pathogen *R. solani*. Analysis of CF using GC-MS and LC-MS techniques revealed the presence of volatile and water-soluble compounds, respectively [9 & 10] (Nadana,et al., 2019; 2020).

3. OBJECTIVES

- To identify mitogenic compounds from the rhizome of *Acorus calamus*. Linn. by using earthworm *E.eugeniae*.

- To screen antimitotic compounds from pseudo-stem *Musa paradisiaca* Linnby using earthworm *E.eugeniae.*

- Molecular docking and simulation studies to support the role of predicted antimitotic compounds from *M. paradisiaca* with important proteins in mitotic cell division

- To determine the importance of the CF of earthworms in plant tissue culture medium with tobacco explants

4. RESEARCH GAP

It is a tremendous challenge for basic preclinical and clinical research to evaluate anticancer or antimitotic drugs. However, because of ethical, medical, and economic limitations, most of the research has to be done in experimental systems. Over decades, researchers have developed well-proven, reliable *in vitro* and *in vivo* methods to evaluate drugs. For most anticancer agents, the initial step of evaluation method involves using of cell culture technique. The use of these standardized experimental methods is time-consuming and costly. Given that it is practically impossible to test large quantities of new unknown potential compounds in vivo.The most important function of in vitro experiments is to select promising candidates for further investigation. To address this problem, new simple *in vivo* animal models are necessary to screen many compounds with limited facilities and expense. We developed such model here with earthworm *E. eugeniae*, by exploiting its regeneration ability from amputated region.

India is one of the largest banana-producing countries, with global production exceeding 153 million metric tons in 2020. Post-harvest, pseudostems and stalks are often considered waste, which can be used for various purposes like organic fertilizer, animal feed, biogas production, fiber production, or bioplastics. However, stems are often burned after harvesting. Research is necessary to find ways to use banana plant waste, such as developing new products or extracting valuable compounds. The idea for this research was initiated by absence of growth of other plants near the banana plant. In order to detect the presence of antimitotic compounds, banana pseudostem was chosen. We wanted to extract antimitotic compounds with water rather than organic solvents. Moreover, using the earthworm regeneration ability, mitogenic compounds could be identified. Hence, *A. calamus* rhizomes were used to identify the mitogenic compounds in our laboratory.

Earthworms create various products that are beneficial for the environment and agriculture. Vermicompost, a natural fertilizer made from organic waste materials, is nutrient-rich compost that improves soil fertility and supports plant growth. Vermiwash, which is a byproduct of earthworm activity, contains plant growth hormones, enzymes, vitamins, and helpful microbial populations. Earthworm castings, which are the digested organic matter excreted by earthworms, are packed with essential nutrients and microorganisms, making them powerful soil conditioners and fertilizers. Earthworm tea, made by soaking earthworm castings in water, is a nutrient-rich liquid that can be used as a natural plant fertilizer or applied as a foliar spray. The coelomic fluid (CF)of earthworm has been recently reported to increase the growth of seedlings as well as induced resistance against plant pathogens (Nadana et al., 2019; 2020). The importance of CF in plant tissue culture medium is not yet studied with model plants. Hence, effect of CF in plant tissue culture medium is studied with the model plant, *Nicotiana tabacum*.

5. MATERIALS AND METHODS

5.1. Onion root tip assay:

This is a widely used technique for preliminary screening of antimitotic compounds. Root tip has actively growing cells and they can be seen in light microscope after staining the cells. The onion root tip assay offers a combination of simplicity, visibility, and versatility, making it a valuable tool for researchers and educators interested in studying cell division and cytogenetics.

5.2. Earthworm regeneration assay:

Amputated earthworm species,*E. eugeniae* can regenerate its body parts when it retains clitellum region was shown in Figure 1. Anterior part of the earthworm (the head region) has clitellum region. This earthworm, known for its long, tube-shaped body, can regenerate from amputated region, especially from the clitellum end. Clitellum region generally starts from the segment 13 to 17, which plays a major role in both reproduction and regeneration. In all our experiments, earthworms were cut at the 30th segment from the mouth and the posterior part is amputated (anus region). The wound healing process generally takes 24 hours. Once the wounds are healed, the compounds to be tested are injected between the clitellum and amputated region.Blastema (new cells) is normally developed after 3 days at amputated region when injected with sterile distilled water (control). Blastema development was inhibited if there were antimitotic compounds in the injected sample. Blastema development was enhanced if there were mitogenic compounds in the injected sample. The following diagram represents the major regions of earthworm and where the amputation was done and used for the assay.

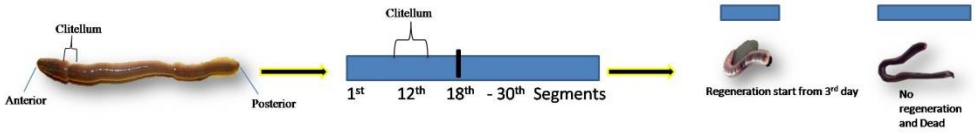

Figure 1. Earthworm regeneration assay

5.3. GC-MS

GC-MS is a powerful analytical technique that combines gas chromatography (GC) and mass spectrometry (MS) to identify and quantify compounds in a sample. GC involves vaporizing a sample and injecting it into a chromatograph, which separates components based on their ability to partition between a stationary phase and a mobile phase. Different compounds have different affinities for the stationary phase, resulting in their separation. MS, on the other hand, involves ionizing compounds in a mass spectrometer, separating them based on their mass-to-charge ratio. The detector measures the abundance of these ions, generating a mass spectrum that provides information about the molecular structure of the compounds. This method is widely used in fields such as environmental analysis, food safety, forensics, and pharmaceuticals, particularly for detecting trace amounts of substances in complex mixtures.

5.4. *In vitro* cell culture assay

In vitro cell culture assays are controlled laboratory experiments conducted outside of living organisms to study cell behavior, responses, and interactions. They are commonly used in biological and medical research for drug development, toxicity testing, and understanding basic cellular processes. Key steps include establishment of new cell culture in fresh nutrient rich medium followed by a treatment or exposure, incubation, and analysis. Cells are isolated from tissues or obtained from cell lines and cultured in a suitable environment. Treatments include drugs, toxins, or experimental compounds, observing their effects on cell growth or behavior. Incubation allows researchers to observe changes in cell morphology, proliferation, viability, and other cellular responses over time. Various techniques are employed to assess the effects of the treatment, including microscopy, cell counting, gene expression analysis, and protein quantification. Data interpretation helps draw conclusions about the effects of tested substances on cells, providing a controlled and reproducible environment for investigating cellular processes and responses.

5.5. LC-MS

Liquid Chromatography-Mass Spectrometry (LCMS) is an analytical technique that combines the separation capabilities of liquid chromatography (LC) with the detection and identification capabilities of mass spectrometry (MS). It is widely used in various scientific disciplines, including chemistry, biochemistry, pharmaceuticals, environmental analysis, and forensic science. LCMS works by injecting a liquid sample into a chromatograph, which separates components based on affinity. The ions are then accelerated and separated in a mass spectrometer,

producing a mass spectrum. This information helps researchers predict and quantify components in the sample.

5.6. Molecular Docking and simulation

Molecular docking and simulation are computational techniques used in structural biology and drug discovery to study interactions between molecules like proteins and ligands. These methods help researchers understand binding mechanisms and predict the behavior of molecules in biological systems. Molecular docking predicts the preferred orientation and binding affinity of a ligand with a protein molecule. Molecular dynamics simulation study simulates the movements and interactions of atoms and molecules over time. Both methods contribute to advancing our understanding of molecular interactions, aiding drug discovery, and developing new therapeutic agents.

5.7. Salkowski test

The Salkowski assay is a method used to detect the presence of indole-ring based compounds in any given sample. If indole-ring containing compound is present, the intensity of the color change is proportional to the concentration of such compounds. This assay is commonly used to identify bacterial species that synthesize such compounds. Bacterial species that can synthesize Indole acetic acid (IAA), plant root promoting hormone, can be identified by using this test.

6. RESULTS AND DISCUSSION

6.1. Identification of mitogenic compounds from *A.calamus*

In our lab, we previously investigated the presence of antimitotic effects from aqueous extract of*A. calamus* rhizome, using different assays. The results showed that aqueous extract contains compounds with antimitotic properties. The crude residue was further fractionated using five different organic solvents viz. benzene, chloroform, ethyl acetate, ethanol, and methanol. We tested these fractions using an earthworm regeneration assay. The methanol and ethyl acetate fractions hindered blastema development, suggesting the presence of potential antimitotic compounds(Rajamanikkam et al 2019b). However interestingly, the ethanol fraction induced blastema development very well, indicating the presence of mitogenic activity. The compounds in ethanol extract likely support stem cells in the clitellum region for the development of blastema. This ethanol fraction also induced mitotic index in onion root tip cells. It indicates the resemblance between the plants and animals in mitosis. The overall outline of identification of mitogenic compounds was shown in Figure 2.

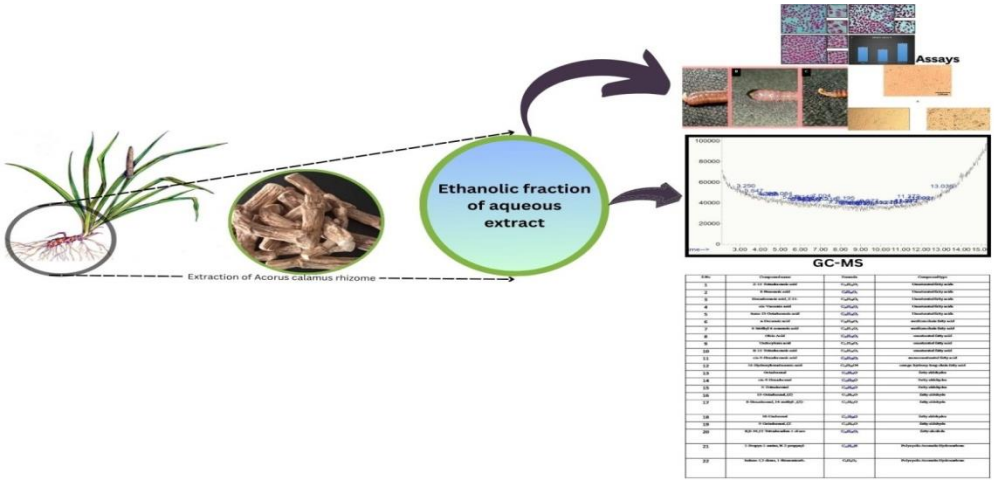

Figure 2.Outline of extraction and identification of mitogenic compounds from *Acorus calamus* Linn. rhizome

6.2. Ethanol fraction had fatty acids and fatty aldehydes:

GC-MS analysis of this ethanol fraction (from aqueous extract of *A. calamus* rhizome) showedthe presence of 38 different compounds.Interestingly, 12 different fatty acids, 7 different fatty aldehydes and onefatty alcohol were found with other 18 different hydrocarbon relatedcompounds.

Our results suggested that these fatty acids and fatty aldehydes in unspecified way facilitated the development of blastema from the amputated earthworms. Amputated earthworms cannot consume anyfood materials as they do not have the posterior part of the body including the anus. These fatty acid and hydrocarbon related compounds in ethanol fractionation could have been used as an energy source by earthworms.Besides; these fatty acids might have been used to synthesize structural lipids in the organelles and cell membranes, as they are needed in the dividing cells (De Carvalho and Caramujo, 2018).

6.3. Antimitotic compounds from banana pseudo-stem

To identify putative antimitotic compounds, the pseudostem of banana plant (PSBP) was chosen and assays were carriedout with aqueous extract of PSBP.Here, weexamined the pith from the pseudostem of the banana plant(PSBP) of *M.paradisiaca* to ascertain the compoundswith antimitotic potential.Plant-based *in vivo* assay with onion roots was carriedout initially. Colchicine, a known commercially available antimitotic compound wasused as a control. Root tips treated with colchicine displayed more number of cells with metaphasedue to its binding withtubulin protein. Similarly, aqueous extract of PSBP-treated root tips reduced mitotic index, indicating that it possessed antimitotic compounds.Besides, this aqueous extract of PSBP also inhibited blastema development in amputated earthworms even after 7 days, whereas blastema development was observed in 3 days from water-treated earthworms. Earthworms injected with colchicine did not exhibit blastema growth. The PSBP extract hindered blastema growth in the healed area, suggesting its influence on mitotic cell division. To further confirm the presence of

antimitotic compounds, an MTT assay with the MCF-7 human breast carcinoma cell line was performed. It demonstrated a significant decrease in cell count and size compared to water-treated cells. The IC50 value of this extract was determined as 56.37 µg/ml. The results in onion root tip assay, earthworm regeneration assay, and MTT assay on human cancer cells provided validation for the presence of antimitotic compounds in the PSBP extract.

The LC-MS analysis of PSBP aqueous extract predicted eight major compounds, out of 435 detected. Three putative antimitotic compounds, α-tocotrienoxyl radical, 1,2,4-nonadecanetriol, and 3′,4′,7-trihydroxyisofavone, were predicted based on their mass value, out of those 8 major compounds. Compounds, similar to tocotrienols, tocopherols, or flavonoid classes, have already been reported to have anticancer activities; however, their modes of action in cell division are unknown.

The α-tocotrienoxyl radical is a form of alpha-tocotrienol, a form of vitamin E, is known for its antioxidant properties. It is formed when alpha-tocotrienol loses an electron, resulting in a reactive species with an unpaired electron. This radical contributes to the antioxidant capabilities of tocotrienols by neutralizing free radicals and reducing oxidative stress. Antioxidants like α-tocotrienol are crucial for maintaining cellular health and protecting cells from reactive oxygen species damage.

1,2,4-Nonadecanetriol is a triol with three hydroxyl functional groups. It belongs to the alcohol class and its biological properties depend on its interactions with biological systems, including solubility, reactivity, and structural features.

3',4',7-Trihydroxyisoflavone is a type of isoflavone, a subclass of flavonoids found in legumes like soybeans and chickpeas. These compounds have antioxidant properties and may contribute to health benefits. Isoflavones, have been studied for potential health benefits, including antioxidant properties, hormonal effects, cardiovascular health, and bone health.

The key difference between previously reports and our data is, our results show the combined effect of all these three ligands rather than individual effect of a ligand in cell division. Moreover, we examined the combinatorial effect of these three ligands with major cell cycle or cell division proteins viz. α-tubulin, β-tubulin and CDK-2 (cyclin dependent kinase-2) proteins by using in-silico studies.

Tubulin is a crucial protein in cell division, particularly in mitosis and meiosis processes. In mitosis, tubulin is crucial for the formation of the mitotic spindle fibers, a dynamic structure that segregates chromosomes. The spindle ensures proper alignment and separation of chromosomes, with microtubules attaching to kinetochores to ensure each daughter cell receives the correct number of chromosomes. Tubulin, a protein with two main isoforms, alpha (α) and beta (β), is crucial for cell division and chromosome segregation. Beta tubulin is targeted by

cancer chemotherapy drugs like taxanes and vinca alkaloids, which disrupt microtubule dynamics.

Cyclin-dependent kinase 2 (CDK2) is another crucial enzyme in cell division, act by binding with specific cyclin proteins at different stages of the cell cycle. CDK2 forms a complex with cyclins, specifically cyclin E during the G1 phase and cyclin A during the S phase. CDK2-cyclin E complexes regulate the transition from G1 to S phase, while CDK2-cyclin A complexes control DNA replication by phosphorylating proteins involved in DNA synthesis. We have chosen these three proteins because they play major roles in mitotic cell division and their X-ray crystallography reports are available.

6.4. *In silico* analysis:

PyRx is open-source software, which is generally used for virtual screening in computational drug discovery. PyRx enables researchers to perform tasks such as virtual screening, analysis, visualization and help researchers to explore potential drug candidates and their interactions with target proteins, aiding in the identification of promising compounds for further experimental validation. Autodock suite, which is part of PyRx software, was used in our molecular docking studies. Results of molecular docking were used in software, Discovery Studio visualizer, to get better images of binding interaction between the protein and ligands.

Molecular docking studies were conducted with three putative antimitotic compounds (ligands), with major cell division proteins like tubulin (α and β) and CDK2, to determine their mode of action. Our study demonstrates the interaction between the three ligands and these proteins. Three different putative antimitotic compounds such as α-tocotrienoxyl radical,1,2,4-nonadecanetriol and 3′,4′,7-trihydroxyisoflavone will be referred here asligand 1 (L1-ATT), ligand 2 (L2-NAT) and ligand 3 (L3-THIF) respectively.

Interactions with α-tubulin: We found that these three ligands formed 9 hydrogen bonds and 11 pi-alkyl interactions with α-tubulin protein. All these three ligands are interacting with 11 different amino acid residues. These 11 different amino acid residues are generally interacting with Mg2++ ions, GTP binding domain or β-tubulin protein during the formation of microtubules. Our results suggest that the interaction of all three ligands with α-tubulin would disturb the formation of dimers with α and β tubulin protein.

Interactions with β-tubulin: Likewise, L1-ATT, L2-NAT, and L3-THIF formed four hydrogen bonds and 15 pi-alkyl interactions with the β-tubulin protein. Three ligands were associated with seven amino acid residues in the β-tubulin protein. Among them one of the amino acid residues, Leu248, had been shown to interact withknown antimitotic compound colchicine. L2-NAT also had binding potential with Thr314, which is another colchicine-binding amino acid residue. Our data suggests

that these three ligands might have influenced the structure of tubulin proteins and disrupted their function during cell division.

Interactions with CDK2 protein: Cyclin-dependent kinases (CDK) are crucial in regulating the cell cycle, and their inhibitors are being used in clinical trials to control abnormal cell division. CDK2 has been extensively studied and used as a target for controlling cell division. Our docking studies showed that all three ligands interacted with different pocket residues in the active sites of CDK2 protein, such as phosphate, hinge, hydrophobic, ribose, and solvent region. L2-NAT formed two H bonds with amino acid residues Kcx33 and Asp145, which are involved in binding with ATP.A CDK2 strong inhibitor, CCT068127, had been shown to make hydrogen bond with Asp145 in the DGF motif, which is important for its phosphorylation activity.

L3-THIF formed one H bond with Kcx33 in CDK2 protein. The hinge region of CDK2 interacted with all three ligands at Ala31 residue. Moreover, amino acid residues of Phe80 and Ala144 interacted with both L2-NAT and L3-THIF at the same hinge region.Eight different amino acid residues in the CDK2 active site interacted with other pockets, indicating high chances of binding these three ligands with the CDK2 protein active site and influencing its activity in the cell cycle.

6.5. Molecular Dynamics (MD) simulation:

It is a computational technique used to study the physical movements and interactions of atoms and molecules over time, widely used in chemistry, biochemistry, physics, and materials science to study bimolecular systems like proteins, nucleic acids, and lipids.

To confirm our results of docking, simulation studies were carried out and found out that the CDK2 protein and α-tubulin protein were the most binding targets for L1-ATT and L2-NAT, whereas L3-THIF was well associated with α-tubulin protein. Atomic MD simulations were performed to explore the dynamic profile of these complexes.The α-tubulin with L3-THIF complex showed more immediate equilibrium status and greater ligand stability. The residues interacting with α-tubulin had lower fluctuation, and the hydroxylic group on the ligand was involved in the polar hydrogen bonding network with Ser140, Asn206, and Asn228, contributing to ligand stability.L1-ATT, L2-NAT, and L3-THIF exerted better affinity during the simulation, with ΔG values of -64.57 ± 4.3 kcal/mol, -62.0 ± 5.0 kcal/mol, and -41.78 ± 7.3 kcal/mol, respectively. Results of simulation studies were aligned with docking studies. Hence, we hypothesize that these three ligands interact with these three ligands and interfere mitotic cell division. The overall outline of identifying the antimitotic compounds from pseudostem of *Musa paradisiaca* was shown in Figure 3.

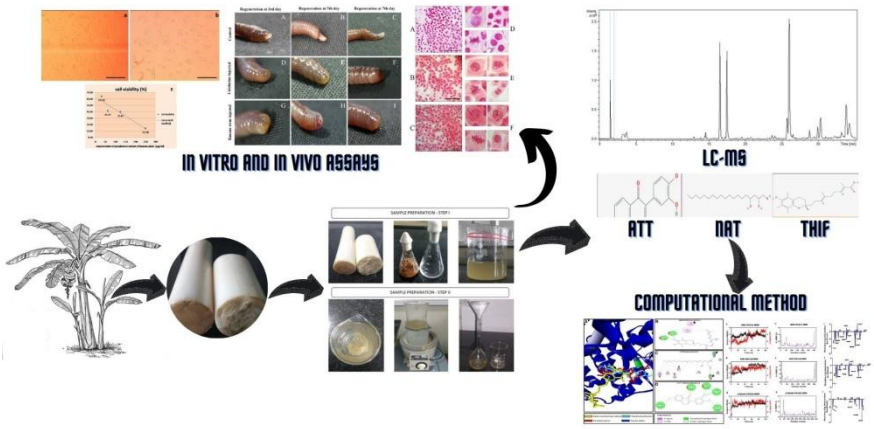

Figure 3.Diagrammaticrepresentation of identifying antimitotic compounds from *Musa paradisiaca* pseudo stem.

6.6. Coelomic Fluid (CF) in Plant tissue culture medium:

The coelomic cavity in earthworm is filled up with coelomic fluid (CF). Interestingly it has been recently reported that this CF has beneficial effect on germination of plant seedlings. Our laboratory has recently reported about the presence of various compounds by using GC-MS and LC-MS studies. The GC-MS analysis suggests that the CF contains volatile compounds, including hydrocarbons and fatty acids like heneicosane, hexadecanoic acid, bis(2-hexyl) ester, tetratetracontane, hen-triacontane, hexatriacontane, tritetracontane, squalene, heptacosane, and thiocarbamic acid. To identify the polar compounds in the coelomic fluid, LC-MS analysis was performed. One of the metabolites, Indole-3-acetyl-L-valine (IAVal) was predicted. These IAA amino acid conjugates are believed to serve as storage or transport forms and are involved in catabolism. However, application of CF of earthworm *E. eugeniae* in plant tissue culture medium is not yet explored.

Nicotiana tabacum, commonly known as tobacco, is frequently utilized for scientific research worldwide. This plant serves as a valuable model organism, offering insights into plant metabolism and defense mechanisms. We used this model plant for all our experiments. Our research focused on the impact of earthworm coelomic fluid (CF), specifically when combined with plant tissue culture medium, on *Nicotiana tabacum* L. stem explants. This fluid effectively induced rapid and robust adventitious root (AR) growth in tobacco stem explants, surpassing the efficiency of synthetic root-inducing hormones like IAA or IBA. Furthermore, our study has identified a potential indole-derivative compound in the CF of earthworms. The importance of indole-derived compounds in AR induction from stem explants has been demonstrated through the use of the amino acid tryptophan. The overview of identifying indole-related compounds from CF was shown in Figure 4.

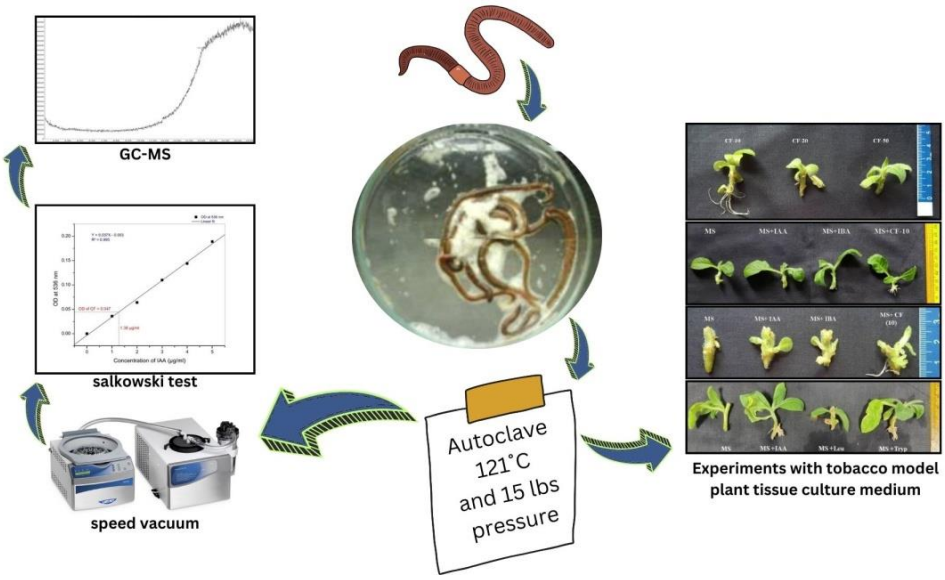

Figure 4.Graphical overview of identifying an indole-related compound from Coelomicfluid

7. CONCLUSION

Fatty acids and its derivatives such as fatty aldehyde or fattyalcohol were predominantly found in the ethanolfractionate from aqueous extract of *A. calamus* rhizomes.We hypothesized that compounds predicted in ethanolfractionate might play asignificant role in induction of cell division in plant and animal tissuesby providing the energy, synthesis of structural membrane lipids in thecell or used as signal molecules. Studies with purified fatty acids or withcombinations of different fatty acids are necessary to understand itsimportance in rapid cell division. Besides, experiments with mammalianmodel organisms might provide the importance of these fatty acids inregeneration of damaged cells.

Regeneration ability of amputated earthworm makesit an excellent system to study the compounds required for cell divisionat molecular level. This system was used here to identify both mitogenic and antimitotic compounds. Mitogenic compounds increase blastema development whereas antimitotic compounds inhibit regeneration from the amputated region. Mitogenic compounds were identified from the medicinal plant *A. calamus*.

We found out that aqueous extract of banana pseudostem (PSBP) contains three antimitotic compounds: L1-ATT, L2-NAT, and L3-THIF. These compounds inhibit cell division in onion root tips, blastema development in amputated earthworms, and breast cancer cell lines. Docking and simulation studies showed that there was strong interaction with key cell division proteins, such as tubulin and CDK2 proteins. Our results suggest that banana pseudostem could be a valuable resource for producing commercially important drug molecules that inhibit cell division.

The coelomic fluid of earthworm *E. eugeniae*is found to initiate adventitious roots (AR) in stem explants of *N. tabacum* when it was diluted ten times with MS medium. In tobacco stem explants, with or without the shoot apical meristem, adventitious root induction was observed after seven days of post-subculture (DPS), whereas commercially available root inducing hormones such as IAA or IBA induced root growth after thirteen DPS. We are reporting first time that the presence of an indole-related compound in the CF of earthworm. GC-MS analysis predicted thatit is 1H-Indole, 5-methyl-2-phenyl compound. Tryptophan, an amino acid with an indole ring, successfully induced AR in tobacco explants after eleven DPS, whereas leucine did not induce AR. We propose that this new putative indole derivative, either alone or in combination with other CF compounds, facilitates the rapid initiation of AR and the growth of tobacco explants.

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Coauthor publications

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